Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion

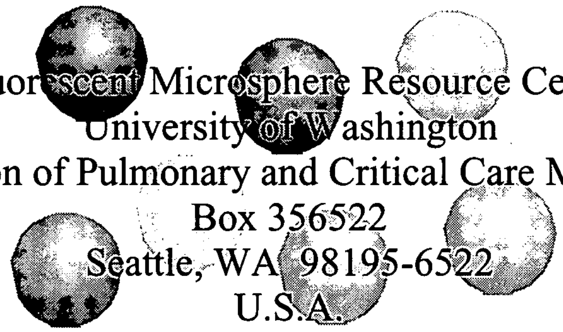

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Table of Contents

SECTION 1: The FMRC

Purpose of the Fluorescent Microsphere Center and this Manual 1-1
  The Fluorescent Microsphere Center 1-1
  The FMRC Manual 1-1
Obtaining Information from the FMRC 1-2
  Subscribing to the Automated Mailing List 1-3
  Mailing to the List 1-3
  Unsubscribing From the List 1-3
  Archives 1-4
References 1-4

SECTION 2: Tutorial on Fluorescence and Fluorescent Instrumentation

Brief Tutorial on Fluorescence 2-1
Introduction to Experimentation 2-3
Fluorescence Instrumentation 2-6
  Perkin Elmer LS-50B 2-6
    The Xenon Source 2-7
    Photomultiplier Tube 2-7
    Determining Fluorescence Ratios (LS-50B) 2-7
    Signal Processing (LS-50B) 2-7
    Slit Settings (LS-50B) 2-8
    Preparation of Sample 2-8
    Sample Temperature 2-9
    Sample pH 2-9
    Sample Exposure to Light 2-9
    Fluorimeter-to-Fluorimeter Variability 2-9
  Other Instruments 2-9
    Hitachi F-2000 2-9
    ISA-Spex FluoroMax-2 2-10
References 2-11

SECTION 3: Standard Curves, Fluorescent Controls, Background Fluorescence & Sources of Error

Standard Curves 3-1
  Construction of Standard Curves 3-2
Fluorescence Controls 3-2
  Making Control Solutions 3-4
  Solvent Blanks 3-5
Organ Fluorescence 3-5
Potential Sources of Error 3-5

SECTION 4: Fluorescent Microsphere Physical Characteristics

Physical Properties 4-1
Storage 4-1
Indications of Deterioration 4-2
Performance Characteristics 4-2
  Purity 4-2
  Microsphere Uniformity 4-2
  Stability 4-2
Spillover of Fluorescence into Adjacent Colors 4-2
Spillover Correction Methods 4-4
Choosing Appropriate Colors for an Experiment 4-5
References 4-6

SECTION 5: Microsphere Measurement of Regional Organ Perfusion

Preparation of Fluorescent Microspheres for Injection 5-1
  Calculation of Microspheres for Injection 5-1
  Estimating the Number of Microspheres per ml 5-2
  Calculation of Injectate Volume 5-2
  Preparation of FMS for Injection 5-2
Reference Blood Flow Sampling 5-3
  Method for Reference Blood Flow Sampling 5-4
Calculation of Regional Perfusion 5-4
  Flow to Each Piece Relative to the Mean 5-4
  Flow to Each Piece in ml/min 5-5
Recovering Microspheres from Samples for Quantification: 5-5
  Blood and Tissue Digestion Followed by Negative Pressure Filtration 5-5
    Digestion of Heparinized Blood for Negative Pressure Filtration 5-6
    Digestion of Solid Tissue for Negative Pressure Filtration 5-6
    Polyamide Woven Filtration Devices 5-8
  Ethanolic KOH, Tissue and Blood Digestion, and Sedimentation 5-8
    Solutions Required 5-8
    Recipes 5-8
    Tubes 5-9
    Taking Samples 5-9
    Tissue Processing 5-10
Quantification of Fluorescent Microspheres 5-11
  Internal Standard Test for Complete Microsphere Recovery 5-11
  Fluorescent Dye Extraction 5-11
    Organic Solvent for Fluorescent Dye Extraction 5-11
    Direct Extraction of Air-Dried Lung Tissue 5-12
    Extraction of Microspheres Following Sedimentation or Filtration Recovery Techniques 5-12
  Fluorescence Measurement 5-12

        Machine Settings 5-12
        Sample Dilution 5-13
        Cuvettes 5-14
        Software/Data Management 5-14
        Wellplate Reader 5-14
        Automated Flow Cell 5-16
    Other Methods for Quantitating Fluorescent Microspheres 5-16
        Direct Counting of Microspheres 5-16
        Flow Cytometer 5-16
        Cryomicrotome/Fluorescent Imaging System 5-18
References 5-20

SECTION 6: Microscopic use of Fluorescent Microspheres

Methods 6-1
    A. Paraffin (Paraplast Plus) Embedding 6-2
    B. 2-Hydroxyethylmethacrylate (Historesin) Embedding 6-3
    C. Vibratome Sectioning of Air-Dried Lung 6-4
    D. Vibratome Sectioning of Unembedded Fixed Tissue 6-4
    E. Vibratome Sectioning of Gelatin-Embedded (Bacto Gelatin) Fixed Tissue 6-5
    F. Frozen Sections 6-6
    G. Staining of Sections 6-7
    H. Coverglass Mounting Media 6-7
    I. Visualization and Photographic Recording 6-10
References 6-11

SECTION 7: Measurement of Regional Alveolar Ventilation with FMS Aerosols

Aerosol Administration System 7-1
Approaches to Maximizing the FMS Signal 7-3
Potential Problems with the Aerosol Administration 7-3
Potential Problems with Analysis of FMS Aerosol Signals 7-3

SECTION 8: Miscellaneous Tips

Miscellaneous Tips Worth Repeating 8-1

SECTION 9: Non-Radioactive Microsphere Bibliography

APPENDICES

List of Suppliers A-1
Contributors to Manual B-1

2 Tutorial on Fluorescence and Fluorescent Instrumentation

Brief Tutorial on Fluorescence

Fluorescence occurs when a molecule absorbs light photons from the u.v.-visible light spectrum, known as excitation, and then rapidly emits light photons as it returns to it's ground state. Fluorimetry characterizes the relationship between absorbed and emitted photons at specified wavelengths. It is a precise quantitative analytical technique that is inexpensive and easily mastered. This chapter outlines the basic concepts and theories on instrument setup and fluorescent dyes in solution.

All chemical compounds absorb energy which causes excitation of electrons bound in the molecule, such as increased vibrational energy or, under appropriate conditions, transitions between discrete electronic energy states. For a transition to occur, the absorbed energy must be equivalent to the difference between the initial electronic state and a high-energy state. This value is constant and characteristic of the molecular structure. This is termed the excitation wavelength. If conditions permit, an excited molecule will return to ground state by emission of energy through heat and/or emission of energy quanta such as photons. The emission energy or wavelength of these quanta are also equivalent to the difference between two discrete energy states and are characteristic of the molecular structure.

Fluorescence occurs when a molecule absorbs photons from the u.v.-visible light spectrum (200-900 nm), causing transition to a high-energy electronic state and then emits photons as it returns to its initial state, in less than 10^{-9} sec. Some energy, within the molecule, is lost through heat or vibration so that emitted energy is less than the exciting energy; i.e., the emission wavelength is always longer than the excitation wavelength. The difference between the excitation and emission wavelengths is called the Stokes shift.

Fluorescent compounds or fluorophors can be identified and quantified on the basis of their excitation and emission properties. Figure 2-1 shows the excitation and emission spectra of a yellow-green fluorescent dye (Molecular Probes, Inc.). The excitation spectra is determined by measuring the emission intensity at a fixed wavelength, in this case 506 nm, while varying the excitation wavelength. The emission spectra is determined by measuring the variation in emission intensity wavelength for a fixed excitation wavelength, in this case 495 nm.

As shown in Figure 2-1, this dye has a detectable emission intensity for a broad excitation range (440-492 nm). Maximum emission occurs at a unique excitation wavelength of 495 nm. Emitted light is detected for a broad wavelength range (492–600 nm), however, when excited at 495 nm, maximum emission occurs at 506 nm. The excitation and emission properties of a compound are fixed, for a given instrument and environmental condition, and can be used for identification and quantification.

The principal advantage of fluorescence over radioactivity and absorption spectroscopy is the ability to separate compounds on the basis of either their excitation or emission spectra, as opposed to a single spectra. This advantage is further enhanced by commercial fluorescent dyes that have narrow and distinctly separated excitation and emission spectra. Table 2-1 lists excitation and emission wavelength pairs which cause maximum emission for 10 common fluorescent dyes.

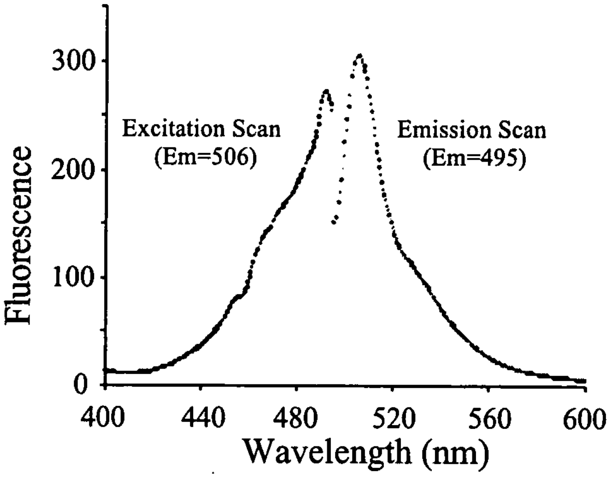

Figure 2-1

Excitation and emission spectra of a yellow-green fluorescent dye (Molecular Probes, Inc.). The Stokes shift of this dye is relatively small (11 nm). In theory, peak intensity of the excitation and emission scans should be equivalent.

Although, maximum emission occur only for specific excitation and emission wavelength pairs, the magnitude of fluorescent intensity is dependent on both intrinsic properties of the compound and on readily controlled experimental parameters, including intensity of the absorbed light and concentration of the fluorophor in solution.

The intensity of emitted light, F, is described by the relationship

$$F = \phi I_0(1-e^{-\varepsilon bc}) \qquad (1)$$

where ϕ is the quantum efficiency, I_0 is the incident radiant power, ε is the molar absorptivity, b is the path length of the cell, and c is the molar concentration of the fluorescent dye (Guilbault, 1990).

The quantum efficiency is the percentage of molecules in an excited electronic state that decay to ground state by fluorescent emission; i.e., rapid emission of a light photon in the range of 200-900 nm. This value is always less than or equal to unity and is characteristic of the molecular structure. The quantum efficiency for some fluorescent dyes is presented in Table 2-1. A high efficiency is desirable to produce a higher relative emission intensity. All non-fluorescent compounds have a quantum efficiency of zero.

The intensity of the excitation light, which impinges on the sample, depends of the source type, wavelength and other instrument factors. The light source, usually mercury or xenon, has a characteristic spectrum for emission intensity relative to wavelength. The properties and advantages of sources are discussed below in the Instrumentation Section.

For dilute concentrations, where $\varepsilon bc < 0.05$, equation (1) reduces to the form:

$$F = k\phi I_0 \varepsilon bc \qquad (2)$$

Table 2-1
Excitation/Emission Wavelengths and Quantum Efficiencies of Fluorescent Dyes in 2-ethoxyethyl acetate

Color	Excitation Wavelength (l_{ex}, nm)	Emission Wavelength (l_{em}, nm)	Quantum Efficiency
Blue	360	423	0.7
Blue-Green	430	467	0.7
Green	445	492	0.7
Yellow-Green	485	506	0.9
Yellow	517	524	0.9
Orange	534	552	0.9
Orange-Red	553	569	0.9
Red	566	598	0.9
Crimson	610	635	0.4
Scarlet	646	680	0.1

If ϕ, I_0, ε and b remain constant, the relationship between the fluorescence intensity and dye concentration is linear (Guilbault, 1990).

At high dye concentrations or short path lengths, fluorescence intensity relative to dye concentration decreases as a result of "quenching". As the concentration of molecules in a solution increases, probability increases that excited molecules will interact with each other and lose energy through processes other than fluorescent emission. Any process that reduces the probability of fluorescent emission is known as quenching. Other parameters that can cause quenching include presence of impurities, increased temperature, or reduced viscosity of the solution media (Guilbault, 1990).

Introduction to Experimentation

A schematic representation of a fluorimeter is shown in Figure 2-2. The light source produces light photons over a broad energy spectrum, typically ranging from 200 to 900 nm. Photons impinge on the excitation monochromator, which selectively transmits light in a narrow range centered about the specified excitation wavelength. The transmitted light passes through adjustable slits that control magnitude and resolution by further limiting the range of transmitted light. The filtered light passes into the sample cell causing fluorescent emission by fluorphors within the sample. Emitted light enters the emission monochromator, which is positioned at a 90° angle from the excitation light path to eliminate background signal and minimize noise due to stray light. Again, emitted light is transmitted in a narrow range centered about the specified emission wavelength and exits through adjustable slits, finally entering the photomultiplier tube (PMT). The signal is amplified and creates a voltage that is proportional to the measured emitted intensity. Noise in the counting process arises primarily in the PMT. Therefore, spectral resolution and signal to noise is directly related to the selected slit widths.

Since source intensity may vary over time, most research grade fluorimeters are equipped with an additional "reference PMT" which measures a fraction of the source output just before it enters the excitation monochromator, and used to ratio the signal from the sample PMT.

Not all fluorimeters are configured as described above. Some instruments employ sets of fixed band-pass filters rather than variable monochromators. Each filter can transmit only a select range of wavelengths. Units are usually limited to 5 to 8 filters and are therefore less flexible. Fiber optics are also employed for "surface readers", to transmit light from the excitation monochrometers to the sample surface and then transport emitted light to the emission monochrometers. This setup has the advantage of speed, but has the disadvantages of increased signal to noise, due to the inline geometry, and smaller path length which increase the probability of quenching.

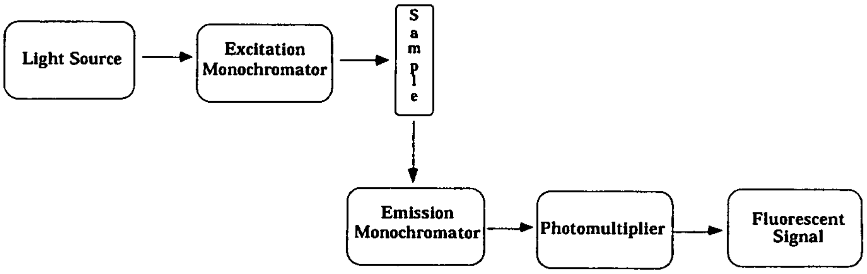

Figure 2-2 Top:

Schematic representation of a fluorescence spectrophotometer. The excitation and emission monochromators are variable band-pass filters.

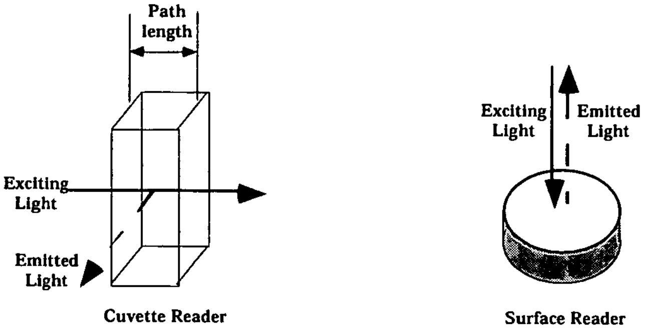

Figure 2-2 Bottom:

Two methods of measuring fluorescence. The cuvette reader excites the sample over the entire path length and reads the emitted light at right angles. The surface reader excites the sample from the top and reads the emitted light returning along the same path direction.

Fluorescent methods have three significant advantages over absorption spectroscopy. First, two wavelengths are used in fluorimetry, but only one in absorption spectroscopy. Emitted light from each fluorescent color can be easily separated because each color has unique and narrow excitation spectra. This selectivity can be further enhanced by narrowing the slit width of the emission monochromator so that only emitted light within a narrow spectral range is measured. Multiple fluorescent colors within a single sample can quantified by sequential measurement of emitted intensity using a set of excitation and emission wavelength pairs specific for each color. The second advantage of fluorescence over absorption spectroscopy is low signal to noise, since emitted light is read at right angles to the exciting light. For absorption spectrophotometry, the excitation source, sample and transmitted light are configured in line, so that the absorption signal is the small difference between the exciting light and the transmitted light, both of which are quite intense. The third advantage is that fluorescent methods have a greater range of linearity. Because of these differences, the sensitivity of fluorescence is approximately 1,000 times greater than absorption spectrophotometric methods (Guilbault, 1990).

A major disadvantage of fluorescence is the sensitivity of fluorescence intensity to fluctuations in pH and temperature. However, pH effects can be eliminated by using nonaqueous solvents, and normal room temperature fluctuations do not significantly affect the fluorescence intensities of commercial dye solutions.

It is important to consider interactions between different types of compounds in a given solution. For instance, one potential problem with neighboring fluorescent colors is that the emitted photons from one compound may cause excitation of a compound that fluoresces at a longer excitation wavelength, causing a reduction in the observed emitted intensity. This would be greatest when the emission wavelength overlaps the excitation wavelengths of two colors. An example is shown in Figure 2-3, where the emission light from the red dye is measured in a solution containing increasing concentrations of crimson dye. It was found that the decrease in red emission intensity was less than 3% for crimson concentrations that were almost three-fold greater than the red microspheres. Potential interactions should be considered when choosing combinations of colors for use in experiments requiring multiple fluorescent microspheres. Future quantification of absorption and emission effects caused by companion dyes will enable us to apply a mathematical correction matrix to reduce a source of error and allow greater flexibility in choice of color combinations.

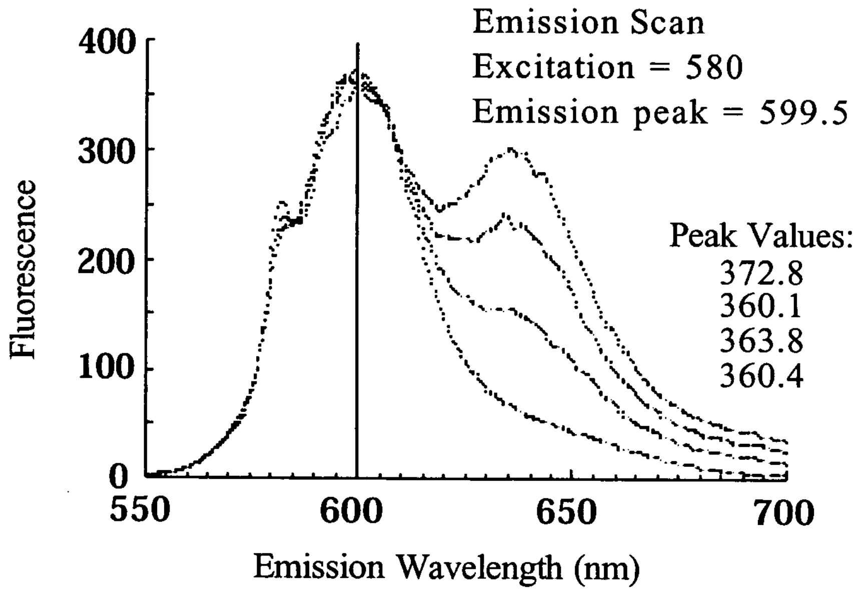

Figure 2-3

Emission spectra of mixtures of red and crimson fluorescent dye (at an excitation wavelength of 580 nm). Per ml of solvent, 2,500 red microspheres were present together with 0, 2,000, 4,000 or 6,000 crimson microspheres. With increasing crimson concentration the reading for red decreases (<3%) due to absorption of the emitted red fluorescence by the crimson (see text). Note that the emission signal at 640 nm (peak of crimson emission) is not read when the sample is excited at 580 nm.

A significant problem can arise when absorption of excited or emitted light from a fluorescent dye occurs in the presence of non-fluorescent substances, significantly decreasing the fluorescence signal and causing erroneous results. Nonfluorescent dyes commonly used for vital stains in tissue samples absorb light from 400- to 680-nm wavelengths. Absorption curves of five commonly used nonfluorescent dyes are shown in Figure 2-4. These dyes significantly interfere with excited and/or emitted light from the dyes in the fluorescent microspheres. Users should check vital dyes to see if they fluoresce and when they do not, assume they can be used in conjunction with fluorescent dyes without considering the absorption characteristics of vital dyes. A simple method is used to determine if a substance interferes with your fluorescence signal. Make a fluorescent sample containing the fluorescent dyes of interest and read them at the appropriate excitation and emission wavelengths. Add the vital dye or other substance to the solution and reread the fluorescence, taking into account any dilution that may have occurred. If the fluorescent signals are the same, your vital dye probably does not affect your fluorescence signal. **Note:** It is best to test this with fluorescence signals and vital dye concentrations in the ranges commonly encountered.

Many fluorescent compounds interact with the excitation light to decompose or otherwise change their structure. To minimize this possibility, an excitation energy of the longest wavelength that results in a detectable fluorescent emission may be more suitable than employing peak excitation energy (Guilbault, 1990).

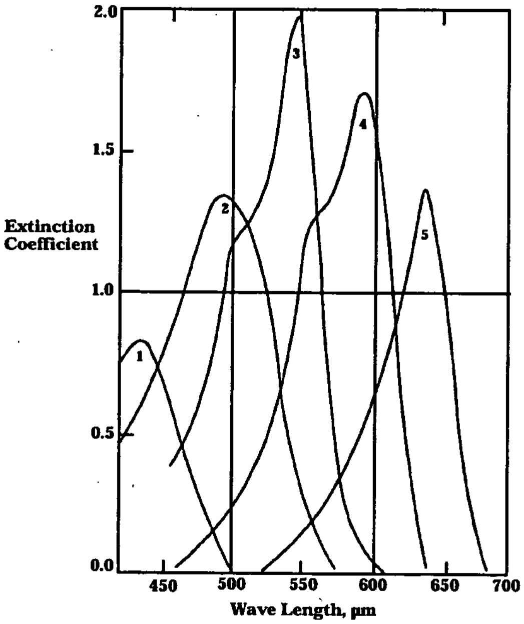

Figure 2-4:

Absorption spectra of five different dyes: 1) Tartrazine (yellow); 2) orange G; 3) fuchsin (red); 4) crystal violet; and 5) Neptune blue BG (patent blue V).

Fluorescence Instrumentation

Perkin-Elmer LS-50B

To obtain accurate data, it is necessary to understand several key aspects of fluorescence spectrophotometry and how these pertain to use of the LS-50B.

All fluorescence instruments contain three basic elements: a source of light, a sample holder, and a detector (Figure 2-2). To be of analytical utility, a system must be equipped with adjustable monochromators that can accurately select excitation and emission wavelengths. It is also essential to monitor and correct any fluctuations in source intensity. The Perkin-Elmer Models LS-50 and LS-50B Luminescence Spectrometer measure the fluorescence intensity of samples in either a continuous scan over a range of wavelengths or at select excitation and emission wavelength pairs. Blood flow analysis data require accurate detection of fluorescence intensities for a sample containing multiple fluorophores. This can be accomplished accurately and rapidly by using a wavelength program that performs serial intensity measurements on a single sample at predefined excitation and emission wavelength pairs.

The Xenon Source

The LS-50B employs a pulsed xenon source that produces a high output using a low voltage, 9.9 watts, resulting in longer lamp life with minimal ozone and heat production. Equally important, the pulsed source reduces potential photobleaching of the sample, during analysis, by several orders of magnitude over continuous sources. The xenon flash lamp produces a 10-µsec pulse of radiation in 16 msec. In fluorescence mode, the photomultiplier tube detector is gated for an 80-msec period in synchronization with the lifetime of the lamp pulse.

Photomultiplier Tube

A photomultiplier dark current is acquired prior to the onset of each lamp pulse and is subtracted from that pulse for correction of phototube dark current. The instrument measures and corrects every flash of the lamp to improve sensitivity at low levels of fluorescence, making it possible to measure samples in room light, thus freeing the user from working through septa in light-tight compartments.

Determining Fluorescence Ratios (LS-50B)

Intensity of emitted light depends on a number of factors, including intensity of incident exciting light (the more powerful the exciting light, the stronger the emitted fluorescence intensity). All light sources currently used in fluorescence instrumentation lack stability over long periods. This causes the output of a xenon lamp to fluctuate as a function of time, which affects measured fluorescence intensity of a given sample, all other conditions being equal. To perform accurate quantitative analyses, these fluctuations must be monitored and corrected to the measured fluorescence intensity. The LS-50B automatically makes this correction by determining the ratios of real-time lamp intensity to sample intensity. The lamp intensity is monitored continuously with a beam splitter to divert a portion of the exciting light to a reference photomultiplier tube (just after it exits the excitation monochromator and prior to entering the sample compartment). The output signal ratio is then scaled and multiplied by a rhodamine correction spectrum stored within the instrument. Comparison with the rhodamine spectrum corrects the wavelength-dependent response of the photomultiplier tubes and the transmission characteristics of the beam splitter. Determining source ratios permits sample fluorescence to be measured free of lamp-related artifacts.

Signal Processing (LS-50B)

When operated in wavelength programming mode, the instrument automatically sets the excitation and emission wavelengths for each dye and dwell time for the specified integration time. The instrument then averages the appropriate number of lamp pulse cycles for the specified integration time. Longer integration time reduces the signal-to-noise ratio for the sample fluorescence intensity. When calculating the optimal sample integration, consider that 1) there are 60 lamp pulses per sec, and 2) the noise in a sample measurement is reduced by the square root of the number of lamp pulses used. Because of the square root relationship between noise reduction and pulse number, there is a point of diminishing return for long integration times. An integration time exceeding 2 sec is necessary only when fluorescence intensities being measured are very small. **Note:** Long integration times (>5 sec) for multi-component samples can result in prohibitively long total analysis time.

Slit Settings (LS-50B)

An important feature of the LS-50B is the availability of continuous variable slit adjustment (0.1-nm increments) on both the excitation and emission monochromators. This flexibility allows the user to fine-tune the instrument for both selectivity and sensitivity in dye discrimination and measurement. The slits can best be described as volume controls for the fluorescence intensity. For optimal instrumental performance, the excitation slit width automatically controls the sample photomultiplier tube voltage. This control provides an optimum signal-to-noise ratio as a function of sample intensity.

In general, a wider slit setting causes higher fluorescence signal measurements. However, because of the fluorescence ratioing system used in the LS-50B (see above), widening the excitation slit width will not increase the reported fluorescent signal ratio, but does increase sample fluorescence signal, resulting in an improved signal-to-noise ratio.

The spectral overlap of dyes used for blood flow analysis can be significant when all available dyes are employed in one experiment; wide slit settings should be avoided in these experiments as they prevent accurate separation of colors. For assays that require five or fewer dyes, the judicious selection of dyes that have minimal or no spectral overlap can be selected (see recommended color combinations in Section 4). As a result, slit settings up to 10-15 nm can be employed for maximum sensitivity. For assays using more than five dyes, care must be taken to insure that a wide slit setting will not result in significant cross-talk between adjacent dyes. With these types of assays there will always be a trade-off between sensitivity and selectivity of dye discrimination.

Solvent and tissue background fluorescent signals can become significant as slit widths are increased. High background signals in the Blue region are frequently seen when using the extraction method with lung tissues. We have no experience with other tissue, but recommend testing all tissue for background fluorescence (see Section 3).

Preparation of Sample

Fluorescence is a very sensitive technique. This is the one criterion that makes it a viable replacement to many radioisotope-labeling procedures. However, it is extremely susceptible to interference by contamination of trace levels of organic chemicals. Potential sources of contamination are ubiquitous since any aromatic organic compound can be a possible source of fluorescence signal. For example, the researcher is a possible source of this type of contamination since oils secreted by the skin are fluorescent. Good laboratory procedure is essential in preventing solvents and chemicals from becoming contaminated with high background fluorescence that could hinder low-level measurements. Solvents should be of the highest level purity obtainable commercially. In addition, care must be taken to eliminate all forms of solid interference (suspended particulates such as dust and fibers). These will float in and out of the sampling area of the cuvette via convection currents, and cause false signals due to light scattering while they remain in the instrument's beam.

Sample Temperature

All fluorophores are subject to intensity variations as a function of temperature. In general fluorescence intensity decreases with increasing temperature due to increased molecular collisions that occur more frequently at higher temperatures. These collisions bleed energy from the excited state that produces fluorescence. The degree of response of an individual compound to temperature variations is unique to each compound. While many commercially available dyes are selected for their temperature stability, care should be taken to eliminate exposure of samples to drastic temperature changes during measurement. If possible, the temperature of instrument's sample compartment should be regulated via a circulating water bath. At lower assay temperatures, higher fluorescence signal will be generated. We have found a 50% decrease in the fluorescence signal of yellow-green microspheres when exposed to 160°C for 15 minutes.

Sample pH

Fluorescence variations due to pH changes are caused by the different ionizable chemical species formed by these changes. The results from these pH variations can be quite drastic since new ionization forms of the compound are produced. For blood flow analysis, the amount of pH variation is dependent on the tissue processing technique. A buffer step has been added to the final rinse for negative pressure filtration technique to minimize pH variations (see Section 5).

Sample Exposure to Light

The fluorescent dyes within microspheres are very stable, losing less then 1% of their fluorescent signal in six months. Once the microspheres are dissolved in solvent, stability decreases. Exposure to sunlight has been shown to significantly degrade dye in less than one week. Therefore we recommend that the samples be stored in the dark both prior to and after extraction.

Fluorimeter-to-Fluorimeter Variability

There is substantial machine-to-machine variability between fluorimeters, even from the same manufacturer. When the same sample is read on two different fluorimeters the fluorescence signals will not necessarily be equivalent. It may be possible to correct for this variability using the internal controls run prior to and during a fluorimeter session (see Section 3). All samples for a given experiment should be read on the same fluorimeter, using identical experimental conditions.

Other Instruments

Hitachi F-2000

The Hitachi F-2000 is another fluorescent spectrophotometer that has been used successfully to separate six different colors of microspheres (Chein et al., 1995). Although its spillover matrix is somewhat greater than the Perkin Elmer machine (Section 4), all colors can be easily separated if the spillover is corrected using a matrix inversion method (solving for a system of linear equations).

ISA-Spex FluoroMax-2

The FluoroMax-2 is a commercially available spectrofluorometer that offers high sensitivity, fast-scanning capability, and selectivity for research and routine fluorescence measurements. The basic components include the source, slits, excitation monochromator, sampling compartment, reference detector, emission monochromator and detector.

The Source

The xenon source that supplies UV performance, is mounted vertically, thereby eliminating sagging of the arc and increasing stability and useful life. The lamp lifetime extends to 1200 hours in the FluoroMax-2 system.

The xenon source is focused onto the entrance slit of the excitation monochromator with an elliptical mirror. Light collection is maximized throughout the system to provide high sensitivity. Besides insuring efficient collection, the reflective surface keeps all wavelengths focused on the slit, unlike lenses that have chromatic aberrations that make them most efficient only at one wavelength.

The Slits

The slits themselves are bilaterally, continuously adjustable from the computer in units of bandpass (wavelength) or millimeters. This preserves maximum resolution and instant reproducibility. The bandpass can range from 0-30 nm depending on the signal strength. For weakly fluorescing samples it is advantageous to increase the bandpass and collect more light. For highly fluorescent samples the narrow bandpass is recommended to avoid exposing the detector to too high signal levels.

The Monochromators

The excitation monochromator is an aspheric design that insures that the image of the light diffracted by the grating fits through the slit. This is an important feature when wanting to measure fluorescence from extremely small sample volumes. The FluoroMax-2 measures high sensitivity regardless of sample volume. The gratings themselves are plane, ruled gratings that avoid the two major disadvantages of the more common concave holographic gratings: poor polarization performance and inadequate imaging during scans that throws away light. The unique wavelength drive scans the grating at speeds as high as 200 nm/s. The grating grooves are blazed to provide maximum light in the UV and visible region.

The Reference Detector

Before the excitation light reaches the sample, a photodiode reference detector monitors the intensity as a function of time and wavelength to correct for any change in output due to age or wavelength.. The photodiode detector has a wider wavelength response range than the older, traditional rhodamine-B quantum counter, and requires no maintenance.

The Sample Chamber

A spacious sample chamber is provided to allow the use of a long list of accessories for special samples, and encourage the user to utilize a variety of sample schemes.

Detector

Emission detector electronics employ photon- counting for low-light-level detection. Photon counting concentrates on signals that originate from fluorescence photons, ignoring the smaller pulses originating in the pmt electronics. The more common method of analog detection adds noise and signal together hiding low signals in the noise.

The emission detector housing also contains and integral high voltage supply that is factory set to provide the maximum count rate, while eliminating most of the dark noise. An optional detector extends the useful range of the system further into the IR.

The Emission Monochromator

All features of the excitation monochromator are also incorporated into the emission monochromator. Gratings are blazed to provide maximum efficiency in the visible.

Computer Control

The FluoroMax-2 is controlled by a PC via a serial link. On start up, the system automatically calibrates and presents itself for either new experiments or stored routines.

The Fluorescence Measurements

The type of scans automatically defined in the sophisticated DataMax software allow the following types of fluorescence measurements:

Excitation
Emission
Synchronous
Time Base Scans
Constant Wavelength Analysis
Multiwavelength Scanning
Single Point Analysis
Recall Experiment

References

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3 Standard Curves, Fluorescent Controls, Background Fluorescence & Sources of Error

Standard Curves

Fluorescence is linearly proportional to dye concentration in dilute samples (see Section 2). However, if the concentration is too great, quenching occurs and the relationship between fluorescence and concentration becomes curvilinear. Standard curves (shown in Figure 3-1) are constructed by analyzing serial dilutions of a single color of microsphere in solvent. They are used to evaluate a spectrophotometer's linearity as well as determine the number of microspheres per ml solvent added to each sample for a given microsphere lot. As shown in Figure 3-1 fluorescence is linear with respect to the number of microspheres in a sample at lower concentrations (generally 2,000 microspheres/ml of solvent) and becomes curvilinear at higher concentrations.

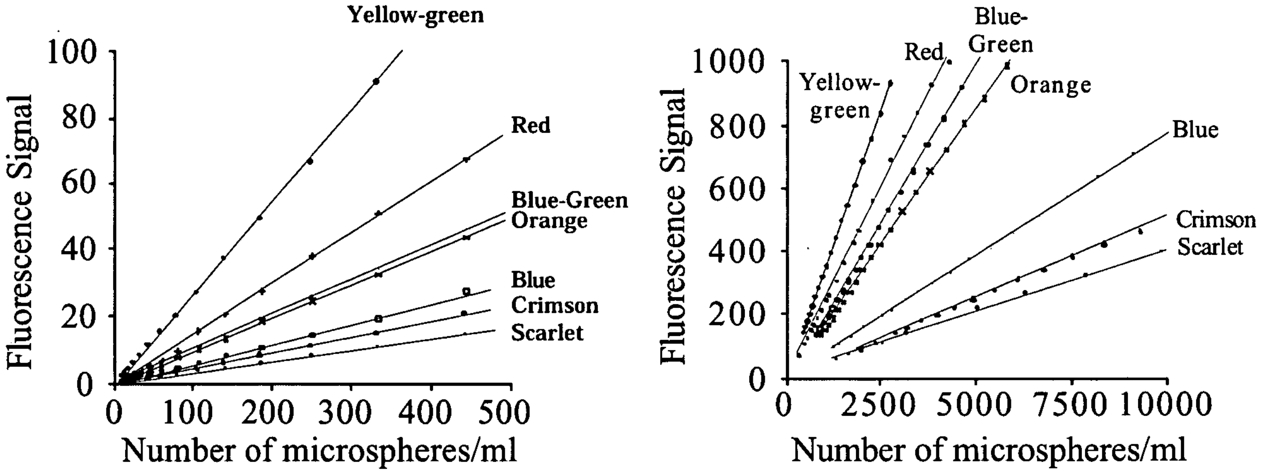

Figure 3-1 Relationship between the fluorescence intensity and the number of fluorescent microspheres per sample.

Each microsphere varies with respect to the quantum efficiency of the dye and the relative "dye load" for that lot of microspheres. These factors affect the fluorescence intensities per number of microspheres. A standard curve should be constructed for each new lot of microspheres to confirm the linearity and concentration range (see below).

Construction of Standard Curves

1) Take 5 µl of 0.2% FluoSpheres® (5,000 fluorescent microspheres) and add it to 10 mls of solvent (we use 2-ethoxyethyl acetate). This yields the fluorescence intensities for ~500 fluorescent microspheres/ml.

2) Depending on the range of your fluorimeter (based on the manufacturer's recommended operating range), calculate the number of µl required to add to 10 mls solvent to yield a fluorescent microsphere concentration that will be just above the upper intensity limit for your fluorimeter.

3) Using this high-stock solution, make multiple serial dilutions of microspheres with very accurate pipetting. Calculate the number of microspheres per ml for each dilution.

4) The fluorescence intensities per number of microspheres per ml are plotted to yield a standard curve for that lot of microspheres.

Fluorescence Controls

Samples of known fluorescence intensities should be measured in the fluorimeter prior to and during the running of a series of samples. Measuring these controls has three important purposes:

1) To make sure that all experimental parameters on the fluorimeter are appropriately set.
2) To check that the lamp and photomultiplier tube of the fluorimeter are functioning properly and the monochromators are appropriately set.
3) To monitor methodological noise.

A change in control intensities may be an early indication that the lamp is beginning to fail or your machine parameters are not properly set.

The best controls would be Lucite-embedded fluorescent dyes that could be used repeatedly. Unfortunately these are not currently available. We routinely make a set of control solutions of fluorescent microspheres dissolved in 2-ethoxyethyl acetate (see the following section on making a set of controls). We read these controls prior to every experimental run and after every 50 samples during a run. Using the internal control function found in the FAC or WINFAC programs (see FAC manual version 8 or higher), a control data file is generated to determine methodological noise. FAC has an automated control check that compares current control intensities to past control intensities stored in the control data file. If intensities differ by more than 5%, a message is sent to the operator that controls are out of range. Each technician should routinely evaluate controls to make sure they do not vary from day to day. Controls are also analyzed at the completion of an experiment to determine methodological noise (see components of methodological noise below). We plot the control intensities and calculate the coefficient of variation (standard deviation divided by the mean). Examples from two of our experiments, as well as "fluorimeter variability" are shown in Figures 3-2, 3-3 and 3-4. Our coefficient of variation (measurement of method noise) runs approximately 2-3% with good operator technique.

Methodological noise measured by the coefficient of variation consists of:

1) Fluorimeter (system) noise (i.e., the reproducibility of intensities without variables such as cuvette washing or cuvette orientation): The LS-50B used in our laboratory has a coefficient of variation of ~1% with the exception of scarlet, which varies up to 2% (Figure 3-2).

2) Other variability: cuvette cleaning, cuvette orientation and cuvette matching. Good technical methods are required to make sure the cuvettes remain clean. If a cuvette is not properly cleaned between samples, the method noise dramatically increases, directly decreasing the confidence in sample intensity. Other sources of operator variability include improper handling of the cuvettes (finger prints/ talcum powder) and particulate material in your sample. Poorly matched cuvettes will dramatically increase coefficient of variation. It is important to use the same set of cuvettes for all the samples from a single experiment.

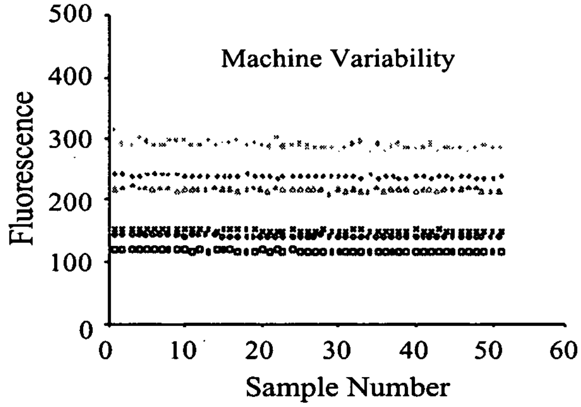

Figure 3-2
Example of machine variability from multiple reading of the control solution without moving the cuvette:

Coefficient of Variation
Blue 0.68%
Blue-Green 0.61%
Yellow-Green 0.63%
Orange 0.61%
Red 0.77%
Crimson 1.07%
Scarlet 2.11%

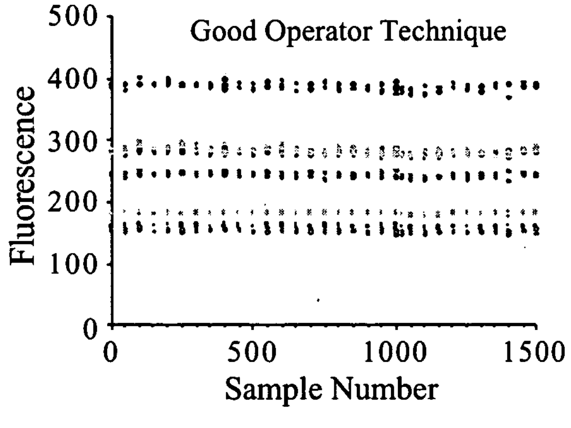

Figure 3-3
Example of a set of controls with good operator technique:

Coefficient of Variation
Blue 1.41%
Blue-Green 1.58%
Yellow-Green 1.57%
Orange 1.37%
Red 1.54%
Crimson 1.70%
Scarlet 1.93%

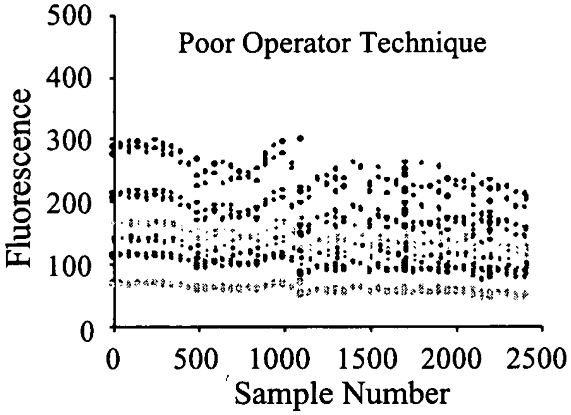

Figure 3-4
Example of a set of controls with poor operator technique

Coefficient of Variation
Blue 11.27%
Blue-Green 11.61%
Yellow-Green 11.99%
Orange 11.58%
Red 10.74%
Crimson 10.71%
Scarlet 10.39%

Figure 3-3 shows a set of controls from a "typical" data set with good operator technique. Figure 3-4 demonstrates a data set with a new technician who was not adequately cleaning the cuvettes between samples. Note the extremely high coefficient of variation.

Making Control Solutions

When making an appropriate set of control solutions, it is important to consider the number of fluorescent microspheres per volume of solvent being used and their "typical" intensities.

1) Control solutions should have a fluorescent signal that is approximately the mean signal for each color in your sample, and be on the linear part of the standard curve, as described earlier.
2) Take 5 µl of 0.2% FluoSpheres® (5,000 fluorescent microspheres) and add it to 10 mls of solvent (we use 2-ethoxyethyl acetate). This yields fluorescence intensities of ~500 fluorescent microspheres/ml and gives a reference intensity for making your control solution.
3) Depending on the number of different colored microspheres being used ("n"), a stock solution is made by dissolving one color of microspheres in 2-ethoxyethyl acetate to yield an intensity that is the "n" times more concentrated than average intensities from an experimental sample.
4) Stock solutions are then combined to yield a control solution in the range of our "typical" sample intensities.

For example, if we use three fluorescent microsphere colors (blue-green, orange and crimson), and our "typical intensities" are 100 for blue-green, 200 for orange and 50 for crimson, we should make a stock solution 3 x 100, or 300 for blue-green, 3 x 200, or 600 for orange, and 3 x 50, or 150 for crimson. The three stock solutions are then combined to yield a 1:3 dilution to make a control solution that reads 100, 200 and 50, respectively, for blue-green, orange and crimson.

Solvent Blanks

Solvent "blanks" are routinely read at the beginning, during, and at the end of each fluorimetry session. This allows for correction of "background" fluorescence to be subtracted from the fluorescence intensity. In our system, 2-ethoxyethyl acetate produces significant background intensities in the blue region only.

Reading solvent blanks also helps assure clean cuvettes. A high blank fluorescence intensity should flag the operator to a dirty cuvette or other problems.

Organ Fluorescence

Tissue can fluoresce causing an increase in fluorescence intensities. With direct extraction of microspheres from air dried lung tissue soaked in 2-ethoxyethyl acetate, there is a significant contribution in fluorescence intensity in the blue region. This tissue fluorescence has an optimum peak excitation and emission pair of 320 and 405- nm, respectively. There is significant background tissue fluorescence only in the blue region. This background tissue intensity can be subtracted from the measured fluorescence intensities.

We have encountered no significant background signal caused by tissue fluorescence in microsphere extractions filtered to remove tissue particulate. Prior to beginning an experiment with new tissue types or recovery methods, we recommend the organ of interest be harvested from an animal that received no fluorescent microspheres, tissue digested, and fluorescence determined for each excitation and emission wavelength pair of interest.

Particulate: It is important that the sample solution be free of particulate, by careful pipetting or filtering prior to analysis. The presence of particulate in solution causes the exciting light to scatter, potentially increasing and/or decreasing sample fluorescence intensities.

Potential Sources of Error

An insufficient number of microspheres per tissue sample will introduce error into the flow estimate. The generally accepted number of microspheres needed for accurate flow measurement is 400/piece (Buckberg, 1971). This problem is solved by increasing the number of injected microspheres.

Under low-flow situations (i.e., small pieces of tissue with very low perfusion having less than 400 microspheres in a given sample), one can determine the relative error of a measurement by the number of microspheres in the reference blood sample and the number of microspheres in the tissue sample (Nose *et al.*, 1985).

Low fluorescence intensities will introduce error into the flow estimates. The lowest acceptable intensities will depend on the sensitivity of the fluorimeter being used. Using a Perkin Elmer LS-50B, the mean of the intensities should be greater than 10 (preferably greater then 50) above background tissue or background solvent intensities.

Microspheres can be lost during the filtering process and/or when transferring the filtered material into the vials in which solvent is added. Microsphere loss will be reduced if the sample can remain in a single vessel during the entire process. Correction for microsphere loss is now presented in Section 5.

If solvent volumes are not precisely reproducible, the measured concentration of fluorescence will not accurately reflect the amount of fluorescent dye per sample. An accurate repeating pipette must be used to add solvent to each sample.

Dilution errors will lead to incorrect estimates of sample fluorescence. Dilutions should be performed with an accurate pipetting technique or by weight.

Samples must be properly stored and handled once the 2-ethoxyethyl acetate has been added. All samples should be stored in the dark until ready to read as light can decay the fluorescence intensities with time. **Note:** Free dye is less stable when not bound in the microspheres; prompt processing of the samples once solvent is added is important.

Quenching of the fluorescence intensities at high concentrations will cause an underestimate of the true amount of microspheres within a sample. Samples with high fluorescence should be accurately diluted until the fluorescence intensities are within linear range of the fluorimeter. The true fluorescence intensities are then calculated from the diluted sample intensities.

Light scattering caused by particles either on the surface of the cuvette or in the solution will lead to incorrect fluorescence intensities. Cuvettes must be cleaned regularly and talc-free gloves worn when handling cuvettes. **Note:** Particulate becomes a greater problem when read at larger slit widths.

Although spillover of one color into an adjacent color is small, error can occur if adjacent color intensities are of disparate magnitudes. This can be avoided by choosing colors that do not overlap or can be corrected mathematically for spillover.

Dye degradation over time. A single dye solution should consist of solvent and the fluorescent color to be used. This solution should be read daily to determine if degradation occurs in specific color/solvent combinations. The solution intensity should be stable for as long as samples are stored in solvent prior to reading.

Fluorimeter variability will introduce error into an experiment if the characteristics of the fluorimeter change over the course of sample measurement from a given experiment. Sources of variability can be the excitation lamp, the photomultiplier tube, or the parameters set by the operator. Measuring the intensity of the known control solution (described earlier) on a routine basis provides the first indication of a fluorimeter change. It is good technique to read all samples from a given experiment within a short period of time (1-2 weeks).

Improper reference blood withdrawal methods will lead to a systematic error (multiplication factor) in calculated piece flows.

Poorly matched cuvettes will dramatically increase your coefficient of variation and decrease accuracy of your measurements. Approximately 40% of the cuvettes we receive are returned because they do not meet our variation requirement of less than 5%.

Cuvette breakage and replacement: If a different cuvette must be used while reading a series of samples from the same experiment, the differences in the cuvette intensities can be corrected by using the percent change in control intensities from initial cuvettes to new cuvettes. Figure 3-5 shows a data set corrected for a new cuvette. Figure 3-5a shows the uncorrected values and Figure 3-5b shows the corrected values.

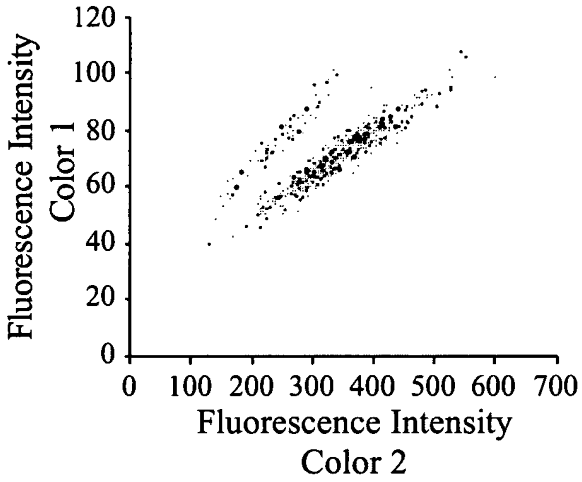

Figure 3-5a: Simultaneous injection of fluorescent microspheres. A cuvette broke during experimental readings and a new set was used to finish the experiment. The points that appear as outliers can be corrected by the percent change in control values.

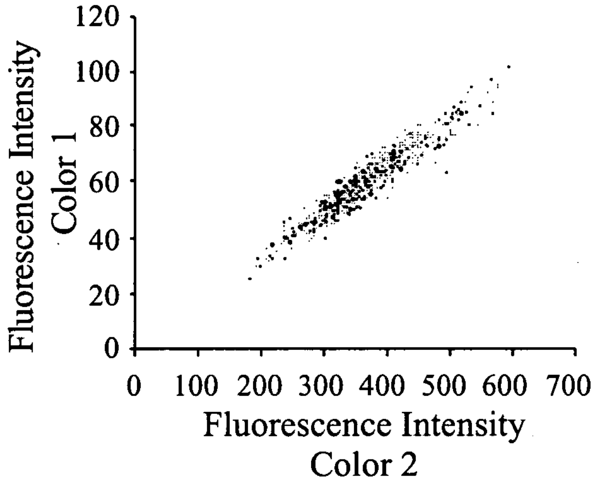

Figure 3-5b: These points have been corrected by the percent change in control values.